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ABSTRACT

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HORMONE PURIFICATION BY ISOELECTRIC FOCUSING IN SPACE

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The objective of our program is the definition and development of optimal methods for electrophoretic separations in microgravity. Our approach is based on a triad consisting of ground based experiments, mathematical modeling and experiments in microgravity. Our methodology differs radically from that of McDonnell Douglas, who base their continuous flow electrophoresis apparatus on so-called zone electrophoresis, while our approach is based on isoelectric focusing.

Zone electrophoresis is a rate process, where separation is achieved in uniform buffers on the basis of differences in electrophoretic mobilities. It is an inherently low resolution method, mainly applicable to separation of cells and cell organelles. In contrast, isoelectric focusing is an equilibrium process, components separating according to their isoelectric points. A pH gradient is necessary, usually also established electrophoretically. Focusing has gained high favor for protein analysis because of its exquisite resolution. Our basic objective is to adapt this technique for large scale protein separation.

Optimization and modeling of continuous flow electrophoresis mainly concern the hydrodynamics of the flow process, including gravity dependent fluid convection due to density gradients and gravity-independent electroosmosis. A background of uniform non-interacting buffer system is generally assumed. Because of the role of gravity in inducing natural convection, the potential benefits of operation in microgravity can be easily assessed.

Optimization of focusing requires a rather different and more complex model describing the molecular transport processes involved in electrophoresis of interacting systems. Our mathematical model represents the first generalized theory of electrophoresis and has been extensively used for computer simulations.

Three different focusing instruments were designed, embodying novel principles of fluid stabilization. Thus, some of the fluid dynamics problems of continuous flow electrophoresis could be avoided. Fluid stabilization has been achieved by (1) flow streamlining by means of membrane elements in combination with rapid fluid recycling; (2) apparatus rotation in combination with said membrane elements; and (3) shear stress induced by rapid recycling through a narrow gap channel.

One of our major unknowns was the assessment of electroosmosis in presence of pH gradients. Experimentally, all ground-based methods suppressing gravity effects also seem to suppress electroosmosis. Microgravity provides a unique opportunity to decouple these two effects, which was the objective of our last space experiment (February 1984). While at first puzzling, the results of the space experiment have led us to the development of a much more comprehensive understanding of electroosmosis.

We now see the classical explanation of electroosmosis with nicely symmetrical laminar flow patterns as only an idealized limited case, restricted to systems of uniform composition at low Reynolds numbers and low electric power. Any departure from these conditions results in a far more complex set of fluid instabilities. This realization has greatly facilitated the design of our latest ground-based prototype instrument. In general, we have directed our space experiments to sharply focused scientific questions, rather than generalized separation instruments.

INTRODUCTION

This space experiment has been rendered possible only through close collaboration with several technicians, scientists and administrators from NASA at Marshall. Unfortunately, time does not permit me to acknowledge all of them, but I must single out Dr. Robert S. Snyder, our science collaborator in this as in all previous space experiments and Mr. Vaughn H. Yost, our program coordinator.

Space electrophoresis was one of the earliest components of NASA's endeavor to develop the basis for a technologic utilization of microgravity. This identification of electrophoresis as possibly benefiting from operations in microgravity was eminently correct. The whole development of electrophoretic methodology has been affected, whether consciously or unconsciously, by the need to neutralize the buoyancy resulting from density gradients. Density gradients, of course, are not always destructive, but can also be utilized constructively as a stabilizing mechanism, as in the original Tiselius electrophoresis apparatus. Gravity can even be utilized as part of the driving force, as in electrodecantation and electrophoresis-convection.

Personally, I was privileged to be part of the NASA effort virtually since its inception. Most of my earlier professional life had been dedicated to preparative electrophoresis, in particular to forced-flow electrophoresis - a variant of electrodecantation. Thus, I was particularly sensitive to the gravity issue. Nevertheless, I had conceptual problems with the efforts to develop zone electrophoresis in space, rather than the more sophisticated techniques of isoelectric focusing and isotachopheresis. A brief explanation of these three variants of electrophoresis is in order.

Zone electrophoresis separates components according to differences in their electrophoretic mobility. It requires a uniform buffer in which the sample is the only discontinuity. It is a rate process, where sample zones broaden with time due to diffusion and sample/buffer interactions without achieving steady state. Because of its inherently low resolution, it is rarely used for protein separations.

Isoelectric focusing separates components according to their isoelectric point. It requires buffers capable of forming a stable pH gradient, established by the electric field. A self-sharpening stationary steady state is achieved irrespective of the starting conditions. It is a high resolution method, widely used for protein and peptide analysis and separations.

Isotachopheresis is a process in which the sample constituents are sorted according to relative net mobilities between a leading and a terminating electrolyte. A steady state is achieved, all sample zones migrating at the same velocity, with contiguous self-sharpening boundaries. While mainly used for the analysis of small molecular weight ions, it offers intriguing possibilities for preparative applications.

RESEARCH APPROACH

The choice between zone electrophoresis and isoelectric focusing will depend to a large degree on the ultimate purpose of the space facility. Zone electrophoresis offers better control of the ionic composition of the buffer and is compatible with separation of living cells. Isoelectric focusing is a more drastic method, where some cells may not survive the treatment. Thus, for cells, zone electrophoresis is the method of choice, while for proteins isoelectric focusing is often preferable.

Our emphasis on isoelectric focusing was predicated by our value judgment that protein and peptide separation presents a more pressing need than cell separations. Mainly, the relation between function and charge is not as clearly established for cells as for proteins. Our emphasis was to a large degree validated by the explosive growth and development of genetic engineering in the last 5 years. This has imposed entirely new demands on separation science. It has also rendered possible the production in recombinant systems of many products which were hitherto derivable only from tissue culture of natural cells. In fact, cloning has replaced the need for cell separations for production purposes.

Furthermore, there may be at present a surfeit of work in continuous flow zone electrophoresis, with independent programs in United States, Soviet Russia, Japan, Germany and France. Some may involve duplication of effort. We believe we are the only group seriously dedicated to the alternate approaches of isoelectric focusing and isotachophoresis in space processing.

The engineering profession has given relatively little attention to electrophoresis, which was largely the province of biochemists. Thus, at the onset of our work there was only a superficial understanding of zone electrophoresis, isoelectric focusing and isotachophoresis. They were treated as separate processes and discussed in models of limited scope. Yet, it was obvious that they are but different aspects of the same process and that they could be described by a single set of equations.

Thus, to advance the understanding of electrophoresis, one of our first efforts was the development of the first general mathematical model based on the fundamentals of the flux due to electromigration and diffusion, conservation of mass and charge, electroneutrality, and solvent/solute interactions due to ionic dissociation equilibria. A computer simulation program has been developed which predicts the behavior of most electrophoretic systems, which are seen to differ only in terms of initial and boundary conditions.

Experimentally, the recycling isoelectric focusing apparatus (RIEF) was constructed, based on a novel recycling principle. Advantage is taken of the fact that in focusing the final steady state is independent of the initial state. Flow stabilization has been achieved by inserting an array of screen elements into the focusing chamber. The fluid dynamics of this apparatus is entirely different from that of the conventional continuous flow zone electrophoresis instrument.

The screen elements, actually nylon monofilament screens of 1 micron porosity, coupled to rapid recirculation, have solved not only the major

problem of gravity driven convection but have also apparently abolished wall electroosmosis. On the other hand, the RIEF has certainly not solved all the problems of effective isoelectric focusing. Thus, there was a strong incentive to develop a focusing apparatus embodying the thin film principle of fluid stabilization employed in the continuous flow zone electrophoresis instruments. The major unknown for the design and optimization of such an apparatus was the role of electroosmosis in isoelectric focusing.

ELECTROOSMOSIS

Classical hydrodynamic treatment of electroosmosis in closed round tubes predicts the development of an axially symmetric laminar recirculation flow pattern with a parabolic flow velocity distribution across the tube. This has been repeatedly verified experimentally through microscope electrophoresis. This involves visual measurement of the migrating velocity of particles in a d.c. electric field at different radial distances of the tube. The velocities describe a parabola, the true mobility of the particle being determined at the so-called stationary level. In flat rather than round cells, the same parabolic profile is observed, though the stationary level is presumed to be located at a somewhat different depth.

In the above measurements, the apparatus is typically filled with a homogeneous buffer, with the electrodes sufficiently far removed from the observation point to preclude changes in electrolyte concentration or composition. Thus, it is also assumed that the electric charge at the walls of the tube is uniform, this so-called zeta potential giving rise to electroosmosis.

Unfortunately, in focusing, the pH gradients affects not only the mobility of the particle but also the zeta potential of the walls of the vessel as well as the local electric field. Thus, microscope electrophoresis cannot yield information on the extent and role of electroosmosis in isoelectric focusing. In fact, all ground-based methods of isoelectric focusing require the abolition of natural convection due to gravity and thus seem also to abolish electroosmosis.

MICROGRAVITY APPARATUS

Microgravity seemed to offer a unique opportunity to decouple gravity driven convection from the electrically driven electroosmosis. An apparatus has been designed for the middeck lockers, incorporating 8 focusing cells, an autonomous power supply and a camera for the recording of the focusing process. Three proteins were to be separated by focusing, HbA and HbC, two genetic variants of human hemoglobin, with isoelectric points at pH 7.0 and 7.35, and human albumin, isoelectric point 4.8. The albumin was stained blue with Bromphenol Blue and the hemoglobins saturated with carbon monoxide for increased stability.

Several constraints had to be observed: cooling of the cells was not practical, thus focusing at low power input had to be adopted. A novel non-gassing electrode system had to be developed, including a palladium electrode for hydrogen adsorption and a palladium-silver half-cell for oxygen adsorption. Two-week stability of the proteins had to be assured,

requiring the addition of bacteriostatic and fungicide agents, as well as pH adjustment. The effect of the long incubation on the palladium electrodes required a great deal of attention.

All features of the space experiment were studied extensively using a ground-based simulator of microgravity. This incorporated a mechanism for rotation of the cell assemblies around their horizontal axis. The continuous change of the direction of the gravity vector is known to permit a degree of convective stabilization.

A number of other variants were to be tested:

Conventional focusing utilizes carrier ampholytes specifically synthesized to generate stable pH gradients. Best known is Ampholine, a product of LKB of Bromma, Sweden. Because such carrier ampholytes are of unspecifiable chemical composition, we have dedicated a great part of our simulation studies to the development of suitable focusing buffers using simple electrolytes of known chemical composition. While Ampholines create a near linear pH gradient, several three component mixture can be utilized to create sharp step-gradients, which will resolve the albumin from the hemoglobins. Because of their sharper concentration gradients they are also likely to exhibit greater convective disturbances. Thus, we decided to test both buffering systems in space.

NASA had developed quite effective anti-electroosmosis coatings, but these were never tested in a focusing arrangement. Thus, the space experiment represented an opportunity to evaluate their effectiveness, though questions regarding coating survival during prolonged incubation in Ampholine were unresolved.

As final design, focusing chambers were tubes, 4.5 cm in length, with an internal diameter of 0.625 cm. Two basic designs were utilized: either a single glass tube of the requisite length, or a series of 14 concentric glass rings, each 0.32 cm in length, for the same total length. These rings could be assembled in three modalities, with monofilament screens, mylar constrictors with an opening of half the internal diameter of the glass rings, or mylar spacers of same internal diameter as the glass rings. All assemblies were housed in plexiglass blocks containing the requisite accessories and assuring leak-proof storage.

The rationale for the screens is their effectiveness for flow stabilization in the RIEF apparatus. They also proved effective in the rotating microgravity simulator. Thus, the cell with screens was most likely to result in good focusing.

The mylar constrictors were used as a test of the hypothesis that disruption of the smooth bore tube with protruding partitions may decrease electroosmosis. This proved to be the case in the microgravity simulator and has been also extensively studied through computer simulation.

The third configuration with mylar spacers of same internal diameter as the glass rings was included as a control for the effects of slicing the glass tube into individual rings.

The final assembly of the eight focusing cells were as follows:

Ampholine-filled:

Cell 1: Single glass tube, uncoated.

Cell 2: Single glass tube, anti-electroosmosis coated.

Cell 3: Rings, separated by non-constricting mylar spacers.

Cell 4: Rings, separated by nylon monofilament screens.

Cell 5: Rings, separated by constricting mylar spacers.

3-component buffer system:

Cell 6: Single glass tube, uncoated

Cell 7: Single glass tube, anti-electroosmosis coated.

Cell 8: Rings, separated by constricting mylar spacers.

All cells were to be focused at 75 volts dc, generated by a set of batteries. The space module had provisions to display the current through each cell. Focusing results in a gradual decrease of conductivity of the solution, thus the solid state ammeters were to give quantitative indication of the progress of focusing. Typically, in good focusing, such as in polyacrylamide gels, the current falls to about 10% of starting value.

Our laboratory has designed and constructed all the cells and furnished to NASA at Marshall, a bread-board arrangement of the final assembly. Marshall has constructed the flight instrument and provided all the requisite testing. Dr. Robert Snyder of Marshall has provided the anti-electroosmosis coating to selected cells and has been our most valuable collaborator throughout the experiment.

PREFLIGHT GROUND BASED TESTING

Preflight testing first required the development of the rotating microgravity simulator. After its satisfactory operation was established, it was also necessary to specify the conditions for the flight. This involved:

1. Development of the two solutions, Ampholine and buffer, offering complete focusing within the operational parameters of the space apparatus (90 minutes at 75 volts), consonant with stability of the proteins during prolonged incubation at room temperature.

2. Conditioning of the palladium electrodes to provide non-gassing service during the entire focusing period. This proved to be a particularly difficult problem.

3. Filling of the individual cells with complete exclusion of air bubbles.

All eight cell configurations were repeatedly tested in the rotator, with results as follows:

As expected, best separation was achieved in the apparatus if filled with a polyacrylamide gel. Not only were all three proteins, albumin, HbA and HbC clearly separated, but there were also visible two smaller bands of other hemoglobin variants. This separation was our standard of comparison.

The cells with the nylon screens showed good separation of albumin from the hemoglobins, essentially each protein occupying one glass ring. The two hemoglobins were not separated.

Second best separations were obtained in the cells with the mylar constrictors. While much more diffuse than in the cells with the nylon screens, separate zones of hemoglobins and albumins were routinely obtainable.

No useful separations were seen in unpartitioned glass tubes due to obvious convective mixing. Antiosmotic coating had no noticeable effect and the buffer solution and Ampholine gave comparable results.

Thus, the space experiment was so designed as to offer the possibility of improved separation at all levels of ground-based performance, as none of the ground-based tests showed the resolution of the polyacrylamide gel.

COMPUTER MODELING OF ELECTROOSMOSIS IN SEGMENTED CELLS

In cooperation with Prof. Saville of Princeton University, a model was constructed predicting the electroosmotic fluid flow with partitions of various geometries. This model has been extensively exercised and has predicted a gain in decreased electroosmosis at constant power. The constrictors obviously cause local recirculating eddies and break up the global recirculation characteristic of plain tubes. The model assumed constant composition and zeta potential as their variation in isoelectric focusing is unknown. The predicted improvement by the model was fully supported by experimental results, but was relatively modest. Unfortunately, the basic problem of the characteristics of electroosmosis in isoelectric focusing remained unsolved.

SPACE RESULTS

The apparatus performed in space as designed, giving us an excellent record of the migration of the colored proteins. Thirty photographs at 2 min. intervals were taken during the first 60 minutes, followed by 10 photographs at 3 min. intervals.

The focusing is best represented by the plots of the changes of measured currents with time. As expected, fastest decrease in current was seen in the cell with nylon screens, followed by the two cells with mylar constrictors. The results, however, were not noticeably better than those obtainable in the microgravity simulator. To the contrary, while hemoglobin was well focused in the screen cell, the albumin was much less well focused than routinely obtainable on the ground. The reason for this is not clear and may have been incidental. Resolution of HbA and HbC was not seen. Thus, while we did not obtain the expected improvement, the partitioned cells did not reveal any unexpected results.

Quite to the contrary, the focusing in the four cells with unpartitioned glass tubes exhibited a completely unexpected behavior. For the first 20 minutes, there was a rapid decrease in current, reflecting good focusing. Shortly thereafter, all four cells (2 with Ampholine and 2 with the 3-component buffer) showed a sudden reversal of the focusing process as

seen from a steadily increasing current. Moreover, the photographic record indicated a coincidental sudden appearance of rapid internal flow - taking the shape of a protruding finger. Considering the 3-component mixture, the reversal occurred between 20 and 22 minutes of focusing in the uncoated cell and 2 minutes later in the coated cell. This delay is a rather good evidence that this disturbance was not due to an external shock to the apparatus, but was caused by internal factors.

POSTFLIGHT ANALYSIS

Two elements of the flight experiment appeared most disturbing: (1) the rapid rate of growth of the protruding 'finger'. The rate was of the order of cm/min, which appeared too fast for electroosmosis; (2) the fact that the mixing, first signaled by the appearance of the finger, continued unabated for the next 70 minutes. Thus, the current through all four cells gradually reached or even exceeded the initial current at the start of the focusing.

Careful review of preflight data failed to reveal similar results. Moreover, despite intense efforts, it proved to be impossible to duplicate either of the two phenomena in a variety of operational modes, whether in the rotating microgravity simulator or stationary operation. It became evident, however, that decreasing the diameter of the tubes improved the focusing process. Thus, with glass tubes of .23 cm diameter or smaller, good focusing was obtained even in unpartitioned tubes, provided rotation is maintained.

In fact, electroosmosis may not have been the only driving force for the convection observed in space. It is certain that the nature of the flow changed in space after approximately 20 minutes of focusing. Protein focusing results in formation of sharp gradients of conductivity as well as of the dielectric constant of the solution. It is well known (e.g., J.R. Melcher and G.I. Taylor, Ann. Rev. Fluid Mech. 1,111,1969) that electric fields can induce electrohydrodynamic body forces due to gradients of the dielectric constant of the medium. This has been observed, however, only in mixtures of immiscible solvents, not in aqueous systems without interfaces.

We are presently evaluating experimentally and through computation this possibility. Experimentally, the effect differs from electroosmosis in two important respects: (1) it is proportional to the square of the imposed electric field, and (2) AC or DC fields are equally effective, provided the gradient is first generated through DC currents. If verified, it would demonstrate a new electrokinetic phenomenon, not previously associated with electrophoresis. It could conveniently account for the observed delay in the onset of turbulence in space, as having been necessary for the formation of the gradient.

This points out the importance of the microgravity experiment which was the first to reveal this effect. We know as yet very little about its scaling when critical dimensions are larger than those used in microscope electrophoresis.

We must assume, however, that the global, well ordered and laminar fluid behavior described in usual textbooks is but a limiting case. Drawing a

parallel between laminar and turbulent pressure driven flows, we can also postulate a turbulent phase of electrokinetically driven flows, whether due to electroosmosis or electrohydrodynamics. Turbulence will be facilitated by several factors: inhomogeneity of composition, large critical dimensions and high electrical field.

To test our hypothesis a series of other cell geometries were prepared for the next space flight, which was supposed to take place in March 1986. The unfortunate Challenger disaster has imposed an unavoidable delay.

POSITIVE FALL-OUT OF THE SPACE EXPERIMENT

As a result of the effectiveness of the microgravity simulator, a new apparatus for ground-based preparative focusing was developed. Dubbed ROTOFOR, it comprises a cylindrical body divided into twenty subcompartments by a parallel array of nylon screen elements. The total capacity of the apparatus is 40 ml. Joule heat is dissipated through a central cold-finger. The resolution is comparable to that obtainable in the RIEF apparatus, but the priming volume is significantly smaller. The patent for the apparatus has been assigned to the University of Arizona and licensed to BIO-RAD of Richmond, Ca.

A second ground-based apparatus has been also completed, this achieving remarkable fluid stability as a result of rapid recirculation of process fluid through a narrow channel between two parallel plates. It is essentially an adaptation of continuous flow zone electrophoresis apparatus to the recycling principle of focusing.

This apparatus is of considerable theoretical interest: it has clearly demonstrated the existence of laminar as well as turbulent electrokinetic flows. At any given recycling rate there appears to be a sharp threshold of electric potential above which the flow becomes turbulent. The shear induced by the rapid flow is essential for fluid stabilization: the higher the shear the higher the threshold. At a high recycling rate and high voltage, the interruption of the flow causes a near explosive outbreak of turbulence.

From a practical point of view, this new apparatus has an advantage over the RIEF and the ROTOFOR since it lacks the screen elements. These are subject to fouling from precipitating proteins. Thus, the new apparatus, referred to as Recycling Free Flow Focusing (RF3) is more tolerant of precipitation and can also be used for the focusing of particulate matter, such as chloroplasts.

FUTURE DIRECTIONS

As a result of the space experiment, two new ground-based instruments have been developed. A far better insight into electrokinetic phenomena has been gained. It remains to be seen if computer modeling will be helpful to predict the change-over from laminar to turbulent flows. Qualitative observations predict that much better focusing will be obtained in space with cells of smaller critical dimensions.

We expect to be scheduled for a second space experiment, where a variety

of new cell configurations will be tested. We will also test whether the presence of proteins is necessary for the onset of turbulence. This is likely, as we know that focused proteins are characterized by much sharper gradients than protein-free zones. We are quite convinced that turbulence is initiated by the presence of these heterogeneities, but microgravity may provide the ultimate proof.

We believe that our results have significant consequence not only for focusing but also for continuous flow zone electrophoresis. Mainly, we have demonstrated that high shear is an effective means for flow stabilization. One of the benefits of space operation is presumed to be the possibility of increasing chamber depth by eliminating gravitational convection. The benefits appear now less clear, as this will decrease shear and may actually destabilize flow.

Primarily, however, we believe that we are still rather far from having truly optimized electrophoresis instruments for either ground-based or space operation. While several of our instruments are quite usable in ground based operation, none of them achieve anywhere near the protein resolution of analytical gels. This is the ultimate objective. We would be less than forthright if we would claim to know how to achieve it, but we are committed to pursue it.

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